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(19)

(54) MICROBIOLOGICAL METHOD FOR ESTIMATING THE TOXICITY OF LIQUID INDUSTRIAL EFFLUENTS

(71) We, THE BRITISH PETROLEUM COMPANY LIMITED, of Britannic House, Moor Lane, London, EC2Y 9BU, a British Company, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed to be particularly described in and by the following statement:—

The present invention relates to a microbiological method for estimating the toxicity of liquid industrial effluents and in particular of refinery effluents.

It is known that certain substances affect the rate of respiration of micro-organisms and that some substances can reduce the respiration rate of yeasts. This reduction in respiration rate has been related to the toxicity of the substances to littoral marine animals e.g. crabs.

We have discovered that certain industrial effluents reduce the rate of respiration of micro-organisms and that when the micro-organisms are in a highly physiologically active state this reduction can be used to estimate the biological toxicity of the effluent.

Accordingly the present invention is a method for estimating the toxicity of a liquid industrial effluent which comprises mixing a culture of an aerobic single cell micro-organism in the exponential growth phase with a measured sample of the effluent in the presence of water, incubating the mixture thus formed under substantially anaerobic conditions to permit toxic substances present in the effluent to penetrate the cells of the micro-organism and to react with the sites for aerobic respiration in the cells, measuring the rate of aerobic respiration of the cells and thereafter comparing the rate of respiration of the cells which have been in contact with the effluent with the respiration rate of cells which have not been in contact with the effluent.

The method is simple and can be operated by relatively unskilled personnel. It is rapid and facilitates the testing of a large number of samples in a day. Consequently it is particularly useful as a day to day monitor of the toxicity of liquid industrial effluents generally and of refinery effluents in particular.

Any aerobic single cell micro-organism can be used as the culture in the present method. The yeasts are particularly suitable because yeast preparations are readily available commercially, e.g. dried or wet bakers yeast or brewers or wine yeast. The yeast *Saccharomyces cereviseae* is the preferred micro-organism because it is universally available.

Techniques for bringing single cell micro-organisms into the exponential growth phase are known and any of these known techniques can be used to prepare the test culture for use in the present method. For example the micro-organism can be cultivated in an aqueous nutrient medium in the presence of a utilisable carbon source and in the presence of a gas containing free oxygen until the culture is in the exponential growth phase. When the micro-organism is a commercial preparation of yeast the cells can be brought into the exponential growth phase by bringing the preparation into contact with an aqueous nutrient medium containing mineral salts and thereafter incubating in the presence of a utilisable nitrogen source e.g. ammonium ions and a utilisable carbon source e.g. glucose and a gas containing free oxygen.

Conveniently the dry weight of microbial cells in the exponential growth phase to be used in the present method can be in the range 0.04 to 2.0 milligrams and preferably 1.0 milligram per millilitre of effluent sample.

The volume of effluent sample required is relatively small for example in the range 1 to 10 millilitres and preferably about 5 millilitres.

The total volume of the mixture containing the culture of microbial cells, effluent sample and water to be incubated is usually in the range 2 to 20 millilitres and preferably about 10 millilitres.

The mixture is incubated under substantially anaerobic conditions until toxic substances present in the sample of effluent to be tested penetrate the cells and react with the sites for aerobic respiration present in the cell. The length of time required can be determined empirically by estimating the time taken to give the maximum effect on the rate of aerobic respiration

of the cells. For example when *Saccharomyces cereviseae* is used as the culture the incubation period is usually in the range 15 to 180 minutes and preferably about 60 minutes. The temperature of the mixture during the incubation period can be in the range 20° C. to 35° C. and preferably is 30° C.

The rate of aerobic respiration of the cells can be measured by any known technique such as for example Warburg Respirometry, gas analysis or by means of radio isotopes. However for the rapid evaluation of samples it is desirable to use a technique which incorporates an oxygen electrode. Any known oxygen electrode can be adapted for use in the present method. Most suitably the oxygen electrode can comprise a chamber having an oxygen permeable membrane which separates the test mixture from two oxygen sensitive electrodes. Preferably the chamber has a means for controlling the incubation temperature of the test mixture, for example a water jacket.

The present invention is further described with reference to the following example.

Example.

(a) Preparation of the culture.

An aqueous nutrient medium having the following composition was made up to 1 litre with distilled water:—

$FeSO_4 . 7H_2O$	83.5	milligrams
$MnSO_4 . H_2O$	20.0	"
$ZNSO_4 . 7H_2O$	253.0	"
$MgSO_4 . 7H_2O$	585.0	"
K_2SO_4	1335.0	"
$CuSO_4 . 5H_2O$	0.66	"
$CaCl_2$	100.0	"
KI	0.1	"
H_3BO_3	0.5	"
$MoO_4 Na_2 2H_2O$	0.2	"
88% H_3PO_4	0.75	millilitres
Conc. H_2SO_4	1.04	"

The pH adjusted to 5.0 by the addition of a concentrated ammonia solution.

8 grams of Vinkwik, a commercially available dried preparation of the yeast *Saccharomyces cereviseae* (Vinkwik is a Trade Mark owned by Southern Vineyards U.K.) was added to 800 millilitres of the aqueous nutrient medium. The mixture was shaken by hand to distribute the yeast and then added to a fermenter vessel. The vessel consisted of a 1 litre glass jar having a stainless steel top plate. The top plate had a pair of wire clamps for attaching it to the jar. The top plate had a central aperture with two roller bearings for a stirrer shaft, an aperture for an air inlet and an exhaust aperture to the atmosphere. The stirrer consisted of a disc type impeller located at the bottom of the shaft. In operation the stirrer gave vortex agitation in the broth. In addition the vessel had a tube for the addition of ammonium hydroxide, and a pH electrode of a conventional type.

Air at a pressure of 5 pounds per square inch was blown into the broth through an open ended pipe having a diameter of about 3 millimetres. The flow rate was regulated by a needle valve, and the quantity measured by a flow meter. The air was filtered by passage through a tube containing cotton wool. The pH electrode was connected to a titrator consisting of a pH meter and a titrant delivery section for the addition of ammonium hydroxide which consisted of an automatic burette coupled with an accumulative recorder for recording the quantity of ammonium hydroxide added.

The fermenter vessel was placed in a water bath maintained at 30° C. An aerobic fermentation was carried out at a stirring speed of 2500 revolutions per minute, an aeration rate of 30 litres per hour, a broth pH of 5.0 and a temperature of 30° C. The pH was maintained at 5.0 by the automatic addition 0.2 N ammonium hydroxide as required. The ammonium hydroxide in addition to controlling the pH provided the nitrogen substrate for the yeast.

The fermentation was continued until uptake of ammonium ions ceased. Growth to this initial stage was thought to be at the expense of the carbon substrate present initially in the dried yeast preparation. One gram of glucose dissolved in 5 millilitres of water was then added to the broth through the exhaust aperture. The fermentation was continued for a few minutes until a rapid consumption of ammonium hydroxide was observed. To prevent foaming at any stage of the fermentation 0.1 cc of a 5 percent weight/volume suspension of Alkaterge C (Alkaterge C is a Registered Trade Mark and consists essentially of a mixture of natural coconut fatty acid) was added as required.

At this stage the yeast culture was in the exponential growth phase. The cells were in a highly active physiological condition and were very sensitive to variation in environmental conditions and in particular factors affecting the rate of cell division. Furthermore this condition could be reproduced and was substantially independent of the physiological state of the yeast in the initial preparation.

(b) Test Procedure.

A series of 5 cc samples of the culture broth containing highly physiologically active yeast cells prepared in accordance with the procedure described in paragraph (a) were added to a series of 25 cc glass test tubes having ground glass necks and glass stoppers. A series of 5 cc samples were taken from four different stages in the purification system of an effluent from a petroleum refinery. A sample of effluent was added to each tube containing a sample of culture broth. Control samples each consisting of 5 cc of distilled water were added to a further series of samples of culture broth.

The following sequence was adopted for the

preparation of the tubes containing control and effluent samples. One control tube was prepared followed by two duplicate effluent tubes. This procedure was repeated for each effluent sample to be tested.

Immediately after addition of the effluent or control samples to the broth sample the tubes were stoppered and shaken by hand for 30 seconds and then placed in a water bath at a temperature of 30° C. and allowed to stand for 60 minutes. The conditions throughout this period were essentially anaerobic. No fermentation activity was observed. It is thought that the toxic substances present in the effluent penetrate the cell and react with the sites for aerobic respiration. The period of one hour is thought to be the optimum time for toxic substances present in the effluent to exert an effect on the yeast. After standing for 60 minutes each tube was shaken by hand for 10 seconds, thereafter the dissolved oxygen content of the suspension was monitored and the rate of aerobic respiration of the culture was measured as follows. The apparatus used was an oxygen electrode marketed by Rank Brothers of Cambridge England. The electrode comprising a water jacketed chamber divided by an oxygen permeable membrane (polytetrafluoro ethylene) to give a zone containing a platinum electrode, a silver/silver chloride electrode and a zone for the liquid to be tested. The liquid test zone contained a magnetic stirrer. The electrodes were connected to a polarising unit and a potentiometric pen recorder.

Immediately after being shaken by hand the stoppered tube containing the mixture was introduced into the test zone of the electrode and the water jacket was maintained at a temperature of 30° C. Air was excluded from the test zone by inserting a stopper in the water jacket which has a small hole for the purpose. The dissolved oxygen content of the mixture was monitored automatically by the electrodes and polarising unit and recorded by the pen recorder. The rate of aerobic respiration of the yeast was measured from the linear portion of the recorded graph of the dissolved oxygen concentration.

The toxicity of each duplicate pair of effluent samples was estimated by comparing the respiration rate of the effluent treated yeast with the mean value of the respiration rate of the control yeast samples.

The results which are given in Table 1 show that there is a decrease in the toxicity of the refinery effluent as estimated by the method of the present invention according to the degree of purification of the effluent.

TABLE 1

		Percentage aerobic Respiration rate in relation to control samples	
	Mean value of control samples	100	
Increasing degree of purification ↓	Effluent treated samples	Sample A	Sample B
	American Petroleum Institute Filtered	29	28
	Sand Filtered	56	58
	Biofiltered	100	100
	Waterfall	96	100

Generally a difference of more than about 5 percent is considered to be significant. Some duplicate samples show greater differences. However the precision is considered to be very satisfactory for a bio assay method.

After an interval of three weeks a further set of four refinery effluent samples were collected from the same points in the effluent stream of the same refinery as those previously described. These samples were tested using exactly the same method as that described for testing the original set of samples. The data obtained is given in Table 2.

TABLE 2

	Percentage aerobic Respiration rate in relation to control sample	
Mean value of control samples	100	
Effluent treated samples	Sample A	Sample B
American Petroleum Institute Filtered 1.	59	58
American Petroleum Institute Filtered 2.	60	63
Sand Filtered 1.	74	76
Sand Filtered 2.	66	77
Biofiltered 1.	111	112
Biofiltered 2.	93	109
Waterfall 1.	111	109
Waterfall 2.	117	112

The data given in Table 2 shows a similar trend of toxicity to that shown by the data given in Table 1. However the general effect was less marked in Table 2. The samples taken from the waterfall showed a stimulatory effect in comparison with the control. It is concluded that the original refinery effluent contained more toxic material than did the effluent three weeks later.

A further test using the same procedure as that described previously in relation to the first set of refinery effluent samples was carried out on a known toxic compound namely pure toluene.

The data obtained from the test is given in Table 3.

TABLE 3

	Percentage aerobic Respiration rate in relation to control samples	
Mean value of control samples	100	
Sample containing Toluene ppm	Sample A	Sample B
100	97	97
200	104	94
350	83	81
500	46	51
750	4.0	4.8

The data shows a threshold toxicity at a toluene concentration of between 200 and 350 parts per million. An article published in Nature 1971 March 5. Vol. 230, pp. 44 to 47 gives the toxicity of toluene to Mosquito fish as 386 to 1,180 parts per million.

WHAT WE CLAIM IS:—

1. A method for estimating the toxicity of a liquid industrial effluent which comprises mixing a culture of an aerobic single cell micro-organism in the exponential growth phase with a measured sample of the effluent in the presence of water, incubating the mixture under substantially anaerobic conditions to permit toxic substances present in the effluent to penetrate the cells of the micro-organism and to react with the sites for aerobic respiration in the cells, measuring the rate of aerobic respiration of the cells and thereafter comparing the rate of respiration of the cells which have been in contact with the effluent with the rate of respiration of cells which have not been in contact with the effluent.

2. A method as claimed in claim 1 wherein the mixture is held for a period of 15 to 180 minutes to permit toxic substances present in the effluent to penetrate the cells of the micro-organism.

3. A method as claimed in either claim 1 or claim 2 wherein the mixture is held at a temperature in the range 20° C. to 35° C. to permit toxic substances present in the effluent to penetrate the cells of the micro-organism.

4. A method as claimed in any one of the preceding claims wherein the dry weight of cells of the micro-organism present in the mixture is in the range 0.04 to 2.0 milligrams per millilitre of effluent.

5. A method as claimed in any one of the preceding claims wherein the volume of the mixture is in the range 2 to 20 millilitres.

6. A method as claimed in claim 5 wherein the volume of effluent present in the mixture is in the range 1 to 10 millilitres.

7. A method as claimed in any one of the preceding claims wherein the micro-organism is a yeast.

8. A method as claimed in claim 7 wherein the yeast is a commercially available preparation of a bakers, brewers or wine yeast.

9. A method as claimed in claim 7 or 8 wherein the yeast is a strain of *Saccharomyces cereviseae.*

10. A method as claimed in claim 1 and as hereinbefore described with reference to the Example.

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